



Mutations within the nuclear localization signal of the porcine reproductive and respiratory syndrome virus nucleocapsid protein attenuate virus replication

Changhee Lee^a, Douglas Hodgins^a, Jay G. Calvert^b, Siao-Kun W. Welch^b,
Rika Jolie^b, Dongwan Yoo^{a,*}

^a Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1

^b Pfizer Animal Health, Kalamazoo, MI 49001, USA

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus replicating in the cytoplasm, but the nucleocapsid (N) protein is specifically localized to the nucleus and nucleolus in virus-infected cells. A ‘pat7’ motif of 41-PGKK(N/S)KK has previously been identified in the N protein as the functional nuclear localization signal (NLS); however, the biological consequences of N protein nuclear localization are unknown. In the present study, the role of N protein nuclear localization during infection was investigated in pigs using an NLS-null mutant virus. When two lysines at 43 and 44 at the NLS locus were substituted to glycines, the modified NLS with 41-PGGGNKK restricted the N protein to the cytoplasm. This NLS-null mutation was introduced into a full-length infectious cDNA clone of PRRSV. Upon transfection of cells, the NLS-null full-length clone induced cytopathic effects and produced infectious progeny. The NLS-null virus grew to a titer 100-fold lower than that of wild-type virus. To examine the response to NLS-null PRRSV in the natural host, three groups of pigs, consisting of seven animals per group, were intranasally inoculated with wild-type, placebo, or NLS-null virus, and the animals were maintained for 4 weeks. The NLS-null-infected pigs had a significantly shorter mean duration of viremia than wild-type-infected pigs but developed significantly higher titers of neutralizing antibodies. Mutations occurred at the NLS locus in one pig during viremia, and four types of mutations were identified: 41-PGRGNKK, 41-PGGRNKK, and 41-PGRRNKK, and 41-PGKKSCK. Both wild-type and NLS-null viruses persisted in the tonsils for at least 4 weeks, and the NLS-null virus persisting in the tonsils was found to be mutated to either 41-PGRGNKK or 41-PGGRNKK in all pigs. No other mutation was found in the N gene. All types of reversions which occurred during viremia and persistence were able to translocate the mutated N proteins to the nucleus, indicating a strong selection pressure for reversion at the NLS locus of the N protein *in vivo*. Reversions from NLS-null to functional NLS in the tonsils suggest a possible correlation of viral persistence with N protein nuclear localization. These results show that N protein nuclear localization is non-essential for PRRSV multiplication but may play an important role in viral attenuation and in pathogenesis *in vivo*.

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an emerged infectious disease of swine and causes significant economic losses to the pig industry worldwide (Albina, 1997). The disease was first recognized in North America in 1987 (Keffaber, 1989) and subsequently in Europe in 1990. Soon

after, PRRS virus (PRRSV) was isolated as the causative agent for the disease in the USA and in The Netherlands (Benfield et al., 1992; Wensvoort et al., 1991). PRRSV has quickly become endemic in nearly all pig-producing countries and is now considered one of the most economically important pathogens of swine worldwide. PRRSV grows preferentially in porcine alveolar macrophages and establishes a persistent infection of up to 5 months in infected pigs (Allende et al., 2000; Wills et al., 1997). Although North American and European isolates of PRRSV cause similar clinical signs and share the same morphology and genome organization, they exhibit significant

* Corresponding author. Fax: +1 519 824 5930.

E-mail address: dyoo@uoguelph.ca (D. Yoo).

genetic and antigenic variations with only 63% nucleotide sequence homology at the genome level. Consequently, PRRSV is divided into two genotypes, the European genotype and North American genotype (Meng et al., 1995; Nelsen et al., 1999; Nelson et al., 1993; Wootton et al., 2000).

PRRSV is a small, enveloped virus possessing a single-stranded positive-sense RNA genome of approximately 15 kb in size (Meulenberg et al., 1993; Snijder and Meulenberg, 1998; Wootton et al., 2000). The genome is 5'-capped and 3'-polyadenylated (Sagripanti et al., 1986; Wootton et al., 2000) and belongs to the family Arteriviridae along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV). The arteriviruses are now re-grouped into the newly created order Nidovirales, together with the Coronaviridae family (Cavanagh, 1997). The PRRSV genome consists of the 5' untranslated region (UTR), nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3 through 7), and the 3' UTR followed by a polyadenylation tail (Meulenberg et al., 1993; Snijder and Meulenberg, 1998; Wootton et al., 2000). ORF1a and ORF1b are translated into the 1a and 1ab polyproteins, respectively, which are then proteolytically processed into 13 non-structural proteins believed to be involved in genome replication and transcription (van Dinten et al., 1999; Wootton et al., 2000; Bautista et al., 2002). ORFs 2a through 7 encode six membrane-associated proteins (GP2a, 2b or E, GP3, GP4, GP5, and M) and a nucleocapsid (N) protein, and these proteins are translated from a nested set of 3'-coterminal subgenomic mRNAs (Meulenberg et al., 1995; Snijder and Meulenberg, 1998; Wu et al., 2001).

The N protein of PRRSV is a small basic protein with an isoelectric point of 10.4 and is comprised of 123 or 128 amino

acids for the North American and European genotypes, respectively (Snijder and Meulenberg, 1998; Wootton et al., 2002). The N protein is the most abundant viral protein in infected cells and constitutes about 40% of the protein content in the virion (Snijder and Meulenberg, 1998). It is highly immunogenic in pigs and believed to be multifunctional (Rodriguez et al., 1997; Wootton et al., 1998). The N protein is a serine-phosphoprotein with unknown functions (Wootton et al., 2002). As the sole structural protein component of the viral capsid, the PRRSV N protein associates with itself by both covalent and non-covalent interactions, providing the basis for viral capsid assembly. The covalently linked N–N homodimerization is formed in the lumen of the endoplasmic reticulum (ER) and the Golgi complex through disulfide linkages via a cysteine residue at position 23 (Wootton and Yoo, 2003). Cysteine 23 of the N protein has recently been shown to be essential for virus replication and infectivity, indicating an essential role of the disulfide-linked N–N homodimerization during virion assembly (Lee et al., 2005).

The entire life cycle of PRRSV occurs in the cytoplasm of infected cells, and accordingly, the PRRSV N protein is predominantly found in the cytoplasm and the perinuclear region. Interestingly, the PRRSV N protein is also found in the nucleus and nucleolus of infected cells (Rowland et al., 1999). Nuclear localization of N proteins has been reported for EAV, which is another arterivirus (Tijms et al., 2002), and for several coronaviruses including avian infectious bronchitis virus, transmissible gastroenteritis virus of swine, and murine hepatitis virus (Hiscox et al., 2001; Wurm et al., 2001). A functional nuclear localization signal (NLS) for the PRRSV N protein has been identified in the stretch of basic amino acids (PGKKKK) at positions 41 to 47 and has been shown to

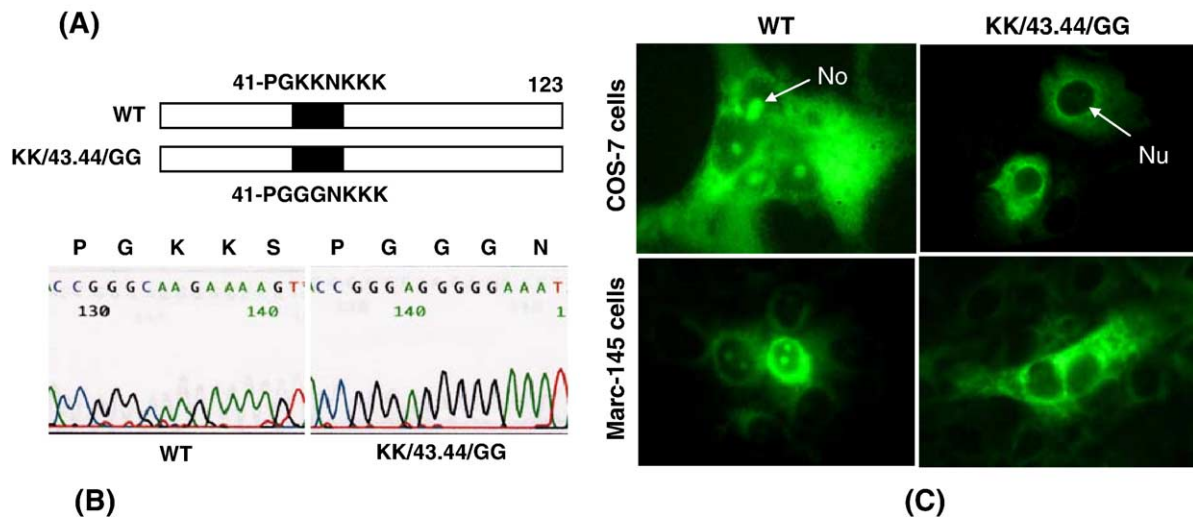


Fig. 1. (A) The 'pat7' nuclear localization signal (NLS, darkened area) of the wild-type N protein and the NLS-null mutant protein. Numbers indicate amino acid positions. WT, wild-type N protein; KK/43.44/GG, lysine substitutions to glycine in the N protein. (B) Electropherogram of the wild-type N gene and the NLS-null N gene. PCR-based site-directed mutagenesis was performed using the wild-type N gene pCi-Neo-N to change codons for lysine at positions 43 and 44 of N to glycine. The N gene was sequenced from the NLS-null N gene. Amino acids are presented in a single letter code. Bold letters indicate substituted amino acids from 'KKS' to 'GGN'. (C) Subcellular localization of N proteins in Cos-7 cells (upper panel) and Marc-145 cells (lower panel) transfected with pCi-Neo-N-WT (left panel) or pCi-Neo-N-KK/43.44/GG (right panel). Gene-transfected cells were incubated for 2 days and reacted with N-specific MAb SDOW17 followed by staining with Alexa green conjugated goat anti-mouse antibody. Nucleus (Nu) and nucleolus (No) are indicated by arrows. Intracellular localization of the NLS-null mutant N protein (KK/43.44/GG) shows the absence of N protein in the nuclei and nucleoli. magnification $\times 40$.

interact with the nuclear transporters, importin- α and - β (Rowland et al., 2003). Therefore, it is believed that the N protein localizes in the nucleus through the NLS-dependent importin α/β -mediated nuclear transport pathway. It is noteworthy that the N protein nuclear localization does not require N–N disulfide-linked dimerization (Lee et al., 2005). Once in the nucleus, the N protein colocalizes and specifically interacts with the small nucleolar RNA-associated protein fibrillarin, implicating an involvement of N in the ribosome biogenesis. Indeed, the N protein has been shown to be able to bind both 28S and 18S ribosomal RNA as well as the viral genomic RNA (Yoo et al., 2003). Since PRRSV assembly and maturation take place in the ER and the Golgi regions, it seems that the PRRSV N protein plays both a structural role in the cytoplasm and a non-structural role in the nucleus and/or nucleolus. The biological relevance of the N protein nuclear localization and its function during infection is presently unknown.

In the present study, a reverse genetics system for PRRSV (Lee et al., 2005) was used to investigate the biological significance of N protein nuclear localization during infection.

An NLS-null mutant virus was generated using an infectious cDNA clone and used to demonstrate that N protein nuclear localization is non-essential for virus replication. Pigs infected with the NLS-null mutant virus exhibited a significantly shorter duration of viremia accompanied by significantly higher titers of neutralizing antibodies than in wild-type virus-infected pigs. The data show strong selection pressure for reversion of the NLS-null at the NLS locus of the N protein.

Results

Substitution of lysine to glycine in NLS and N protein nuclear localization

The North American genotype PRRSV N protein contains two putative NLS motifs at positions 10 to 13 (10-KRKK) and 41 to 47 (41-PGKKN/SKK), and these motifs resemble ‘pat4’ and ‘pat7’ NLS, respectively. Previous work using the PRRSV SDSU-23983 isolate showed that the ‘pat7’ NLS was necessary and sufficient for nuclear localization of N (Rowland

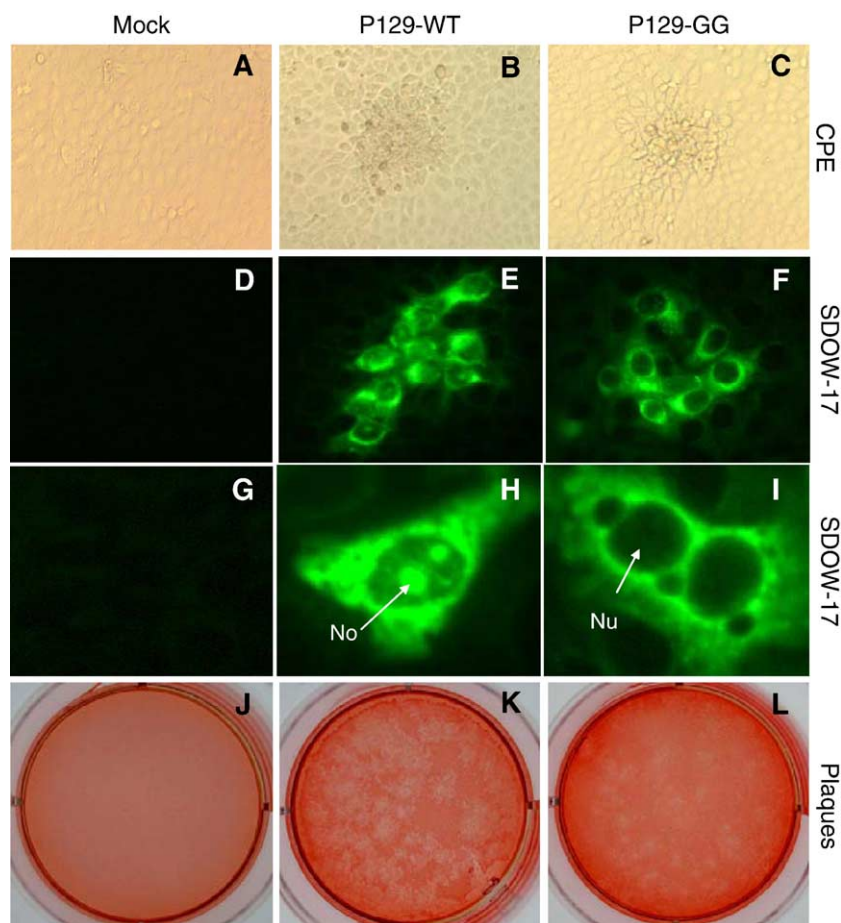


Fig. 2. Infectivity of the P129-GG full-length genomic clone. CPE, cytopathic effects; SDOW-17, monoclonal antibody specific for the N protein. Marc-145 cells were transfected with 2 μ g of P129-WT or P129-GG DNA and incubated for 3 days. CPEs became visible at 3 days post-transfection and were photographed using an inverted microscope. For immunostaining, cells were reacted with SDOW17 at 2 days post-transfection followed by incubation with the goat anti-mouse antibody conjugated with Alexa green. Nucleus (Nu) and nucleolus (No) are indicated by arrows. (D to F) Magnification 20 \times ; (G to I) magnification 40 \times . The intensely stained spots correspond to the wild-type N protein and the NLS-null mutant N protein. The wild-type N protein was concentrated in the both cytoplasm and nucleus, while the NLS-null protein was excluded from the nucleus. Plaque morphology of P129-WT (K) and P129-GG (L). At 24 h of DNA transfection, cells were overlaid with 0.8% agarose and further incubated for 5 days. Plaques were stained with 0.01% neutral red and photographed. Note that the size of plaques for P129-GG virus is small in comparison to wild-type plaques.

et al., 2003). The presence of positively charged lysine residues at 43 and 44 suggested that these two residues might constitute a core sequence responsible for the N protein nuclear localization. Lysines at 43 and 44 of 41-PGKKKK were substituted to glycine, and a 'pat7-modified' mutant N gene was constructed and designated KK/43.44/GG (Figs. 1A and B). The KK/43.44/GG gene was transfected into COS-7 cells, and at 48 h post-transfection, cells were stained with the N-specific MAb SDOW-17. The wild-type N protein accumulated both in the cytoplasm and the nucleus with a distinct nucleolar staining (Fig. 1C), but expression of the KK/43.44/GG protein was restricted to the cytoplasm and perinuclear regions with no staining in the nucleus and nucleolus (Fig. 1C). Subcellular distribution of KK/43.44/GG protein was also examined in Marc-145 cells since this is the established cell line permissive for PRRSV infection. As with COS-7 cells, Marc-145 cells also showed cytoplasmic and perinuclear stainings but absence of staining in the nucleus, indicating that the lysines at 43 and 44 were essential amino acids for the function of the NLS of the N protein.

Generation of NLS-null P129-GG virus

A reverse genetics system was applied to generate NLS-null mutant PRRSV. In this system (Lee et al., 2005), the full-length genomic cDNA is under the control of the cytomegalovirus immediate early promoter. Direct transfection of plasmid DNA initiates a full cycle of virus replication and produces infectious progeny virus. By site-directed mutagenesis, the motif of 41-PGKKSKK in the N gene of the shuttle plasmid was altered to 41-PGGGSKK. Nucleotide sequencing of screened clones verified the presence of the 'GGN' modification in the full-length clone. The 'GGN' mutation in the full-length clone was designated pCMV-S-P129-GG. No additional mutation was found throughout the N gene other than the 'GGN' mutation.

Infectivity of P129-GG

Infectivity of the NLS-null pCMV-S-P129-GG genomic DNA clone was determined first by transfection into Marc-145 cells followed by observation for cytopathic effect (CPE). The wild-type and NLS-null mutant clones induced weak CPE by 3 days post-transfection, and thereafter, the CPE became prominent (Figs. 2B, C). The specificity of CPE was confirmed by immunofluorescence staining using the N-specific MAb SDOW17. Clusters of cells showed bright fluorescence, indicating virus infection and spread of infection to neighboring cells (Figs. 2E, F). In cells transfected with the wild-type clone, the N protein was distributed throughout the cytoplasm and nucleoplasm, showing the nuclear translocation of N of the molecularly cloned virus (Fig. 2H). By contrast, the N protein staining in cells transfected with the NLS-null clone was observed in the cytoplasm and the perinuclear region, but not in the nucleus or nucleolus (Fig. 2I).

To prepare the stock of NLS-null virus, culture supernatants were harvested at 5 days post-transfection (designated 'P129-GG'). Virus titers were determined to be 1×10^2 , 4.0×10^2 , and

5×10^3 PFU/ml for passages one (P1), two (P2), and three (P3), respectively (Fig. 3A). The wild-type (designated P129-WT) virus was generated in parallel from the pCMV-S-P129 clone with virus titers of 1×10^3 , 7.9×10^3 , and 6.3×10^5 PFU/ml, for P1, P2, and P3, respectively (Fig. 3A). Thus, by P3, the NLS-null mutant P129-GG virus had a titer 100-fold lower than that observed for P129-WT. Viral plaques formed by P129-GG were drastically reduced in size, and most plaques were of pinpoint size (Fig. 2L), suggesting a negative effect of the NLS mutation on the rate of cell-to-cell spread during infection.

To confirm whether the NLS mutation was still retained in the P129-GG virus during passages in Marc-145 cells, RT-PCR and sequencing were performed for the N gene using the P3

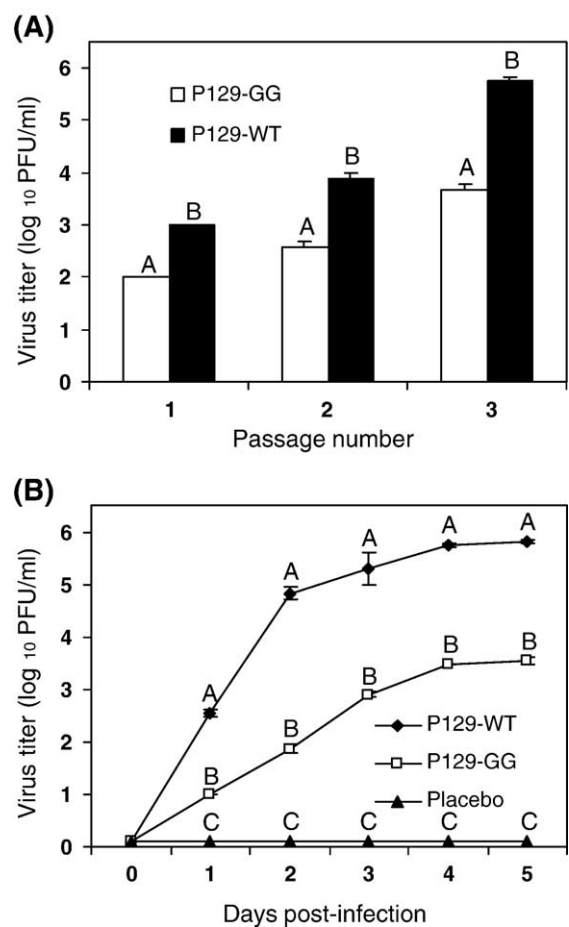


Fig. 3. Growth kinetics of the NLS-null P129-GG virus in Marc-145 cells. (A) Titers of wild-type virus and NLS-null P129-GG virus during the first three passages. The P129-WT and P129-GG viruses were passaged three times in Marc-145 cells and titrated by plaque assay. The titers are expressed in log₁₀ PFU/ml. The culture supernatants from DNA-transfected cells were designated 'passage-1'. Error bars represent standard errors of mean values from two independent experiments. At each passage number mean titers with different capital letters (A, B, or C) differ significantly ($P < 0.05$). (B) One-step growth curves for P129-WT and P129-GG virus. Cells were infected at an MOI of 1–5 with the passage-3 preparation of indicated virus in duplicate. Samples were taken at the indicated times, and virus titers were determined by plaque assay. The results are expressed as mean values from two independent experiments, and error bars represent standard errors of mean values from two experiments. At each day post-infection, mean titers with different capital letters (A, B, or C) differ significantly ($P < 0.05$).

virus. To avoid possible carry-over DNA contamination from transfections during P1 virus production, RNA preparations were treated with RNase-free DNase I. Four hundred-base pair PCR fragments were obtained from both P129-WT and P129-GG, and sequencing results showed stable incorporation of the ‘GGN’ mutation in the viral genome for at least three passages in cell culture.

The one-step growth kinetics of P129-GG was determined using P3 virus. Cells were infected with P3 virus at an MOI of 1 to 5 (Fig. 3B). The wild-type virus reached a titer of 1×10^5 PFU/ml by 3 days post-infection, and the titer increased to a maximum of 6.3×10^5 PFU/ml by 5 days post-infection (Fig. 3B). In contrast, P129-GG showed retarded growth with titers of 7.9×10^2 to 3.2×10^3 PFU/ml during 5 days of infection (Fig. 3B). These data demonstrate that nuclear localization of the PRRSV N protein is non-essential for virus multiplication, but the ‘GGN’ mutation at the NLS locus reduces the growth rate of PRRSV.

Experimental infection of pigs with NLS-null P129 GG virus

To examine in vivo effects of the NLS-null virus in the natural host, a pig infection study was conducted. Following challenge, pigs in the P129-GG and P129-WT groups developed signs of a mild PRRS virus infection, but the severity of clinical signs was similar in the two infected groups. Fever, defined as a rectal temperature higher than 40 °C, developed in four and six pigs in the P129-GG and P129-WT

groups, respectively. Between 3 and 6 days post-infection, pigs infected with P129-WT tended to have slightly higher temperatures than P129-GG pigs (data not shown). Weight gains were numerically higher in pigs infected with P129-GG virus than in pigs infected with P129-WT but did not differ significantly (data not shown).

While animals in the control group remained PRRSV negative throughout the study, pigs inoculated with either P129-WT or P129-GG developed viremia by 4 days post-infection (Table 1). In pigs inoculated with P129-WT, mean duration of viremia was significantly longer than in P129-GG inoculated pigs (8.7 days and 5.7 days, respectively). The peak mean virus titer in serum was higher for P129-WT inoculated pigs than for pigs receiving P129-GG (5×10^1 PFU/ml and 1.3×10^1 PFU/ml, respectively), but the difference was not statistically significant. These data suggest that an NLS-null mutant virus induces a lower viral load and shorter duration of viremia compared to those in wild-type virus-infected pigs.

All pigs infected with P129-GG or P129-WT seroconverted by 10 days post-infection, as evaluated by ELISA (IDEXX Laboratories, Westbrook, Maine) (Fig. 4). Placebo pigs remained seronegative. The mean S/P ratio of antibody for P129-GG pigs was higher than that of P129-WT pigs (2.3 and 1.8, respectively), but the difference was not statistically significant.

In pigs infected with P129-WT, serum neutralizing (SN) antibodies were detected by 14 days post-infection and reached a geometric mean titer (GMT) of 4.3 by 28 days post-infection.

Table 1
Viremia in individual pigs inoculated with placebo, wild-type virus P129-WT, or NLS-null mutant virus P129-GG

Fig ID	Inoculum	Viremia (log ₁₀ PFU/ml) at day post-inoculation							Duration of viremia in days
		0	4	7	10	14	21	28	
27	Placebo	– ^a	–	–	–	–	–	–	0
29		–	–	–	–	–	–	–	0
31		–	–	–	–	–	NA ^b	NA	0
32		–	–	–	–	–	NA	NA	0
39		–	–	–	–	–	–	–	0
41		–	–	–	–	–	–	–	0
42		–	–	–	–	–	–	–	0
Mean ^c		–	– ^A	– ^A	– ^A	–	–	–	0 ^A
26	P129-WT	–	1	1.3	1	–	NA	NA	10
28		–	1.3	1	–	–	–	–	7
30		–	1	2	1	–	–	–	10
35		–	1	1.5	–	–	–	–	7
37		–	0.7	1	–	–	NA	NA	7
40		–	2.8	3	1	–	–	–	10
43		–	1.5	1.7	1	–	–	–	10
Mean		0	1.3 ^B	1.7 ^B	0.6 ^B	0	0	0	8.7 ^B
33	P129-GG	–	0.7	–	–	–	–	–	4
34		–	1.3	2.2	–	–	NA	NA	7
36		–	1.4	–	–	–	–	–	4
38		–	0.7	–	–	–	–	–	4
44		–	1.3	1.6	–	–	NA	NA	7
45		–	1.3	2.7	1.9	–	–	–	10
46		–	1.3	–	–	–	–	–	4
Mean		0	1.1 ^B	0.9 ^{AB}	0.3 ^{AB}	0	0	0	5.7 ^C

^a –, no viremia.

^b NA, not applicable due to early euthanasia.

^c In each column, means with different superscripts (^A, ^B, or ^C) differ significantly ($P < 0.05$).

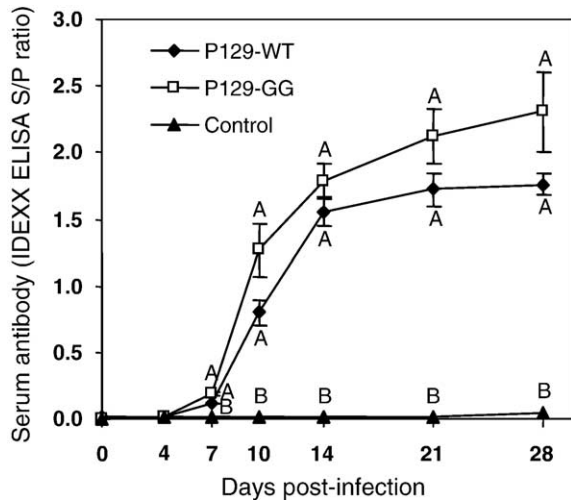


Fig. 4. Serum antibody response for PRRSV in pigs infected with wild-type virus or NLS-null mutant P129-GG virus. PRRSV-specific serum antibodies were determined using the HerdCheck® PRRS ELISA 2XR kit. S/P ratios of greater than 0.400 are considered positive. Error bars represent standard errors of the mean. At each time point, means with different capital letters (A, B, or C) differ significantly (Kruskal–Wallis non-parametric test, $P < 0.05$).

In contrast, neutralizing antibody responses developed faster and higher in pigs infected with P129-GG. SN titers in this group reached a GMT of 9 by 21 days post-infection and increased to a GMT of 18 by 28 days post-infection (Fig. 5). These data suggest that pigs infected with P129-GG virus generate significantly higher neutralizing antibody titers than pigs infected with wild-type virus.

At 14 days post-infection, gross lung lesions were determined in two pigs from each group, and limited areas of atelectasis of lung tissue and varying degrees of lymphadenopathy were noted. Lung lesions did not appear significantly different between pigs from different virus treatment groups. Overall, lung lesion scores in pigs were similar and minimal, regardless of the treatment group (data not shown).

Reversions of NLS-null virus in pig 45

In comparison to values for other pigs within the same P129-GG group, pig 45 showed a higher viral load during viremia (5.0×10^2 PFU/ml vs. maximum mean titer of 1.3×10^1 PFU/ml), lower ELISA S/P ratios (1.002 vs. a mean of 2.757 in other P129-GG pigs at 28 days post-infection), and lower SN titers (5.7 for pig 45 vs. a GMT SN titer of 24.7 for the rest of the group at day 28). Similar values were observed in pigs infected with wild-type virus, suggesting reversion of the ‘GGN’ mutation at the NLS locus. To examine this hypothesis, plaque assays were carried out using Day 7 post-infection serum samples from pig 45. Ten well-isolated individual plaques were picked and subjected to direct extraction of RNA followed by RT-PCR for the N gene and sequencing. Fig. 6A shows the nucleotide and deduced amino acid sequences for the NLS region of N from each plaque. The sequence data revealed that nine

plaques out of 10 were mutated; six plaques mutated to 41-PGRRNKK, 41-PGRGNKK, or 41-PGGRNKK, and three plaques mutated to 43-PGKKSKK (Fig. 6A). Only one plaque out of 10 retained the original NLS-null sequence of 41-PGGGNKK. No other mutation was found elsewhere in the N gene in the ten plaques from pig 45. The mutated amino acids represented an arginine mutation at position 43 or/and 44. Since arginine may substitute for lysine without disruption of the NLS function (Horton and Nakai, 1997), the mutated plaques likely reflect functional NLS reversions (Fig. 7). Reversions could not be found in other pigs in the same group. Similarly, no mutation was found in the N gene of the virus isolated from pigs infected with wild-type virus. The original NLS-null mutant virus was cultivated in Marc-145 cells for 20 passages, and the N gene was sequenced from the passage 20 virus. No additional mutation was found in the N gene. These data suggest strong selection pressure at the NLS locus of N for reversion during replication of PRRSV in pigs.

Virus persistence in tonsils

One of the hallmarks of PRRSV infection is viral persistence in pig tonsils for a prolonged period of time. Numerous reports show that infectious virus may persist for up to 150 days following infection (Allende et al., 2000; Wills et al., 1997). In the present study, tonsils from all pigs infected with P129-GG or P129-WT were positive for the N gene by RT-PCR (Fig. 6B), indicating that all infected pigs harbored the virus in the tonsils for at least 4 weeks post-infection. No mutation was found in the N gene from the P129-WT pigs, and their sequences retained the wild-type NLS motif of 43-PGKKKNKK. Pigs infected with P129-GG, however, all showed mutation of at least one nucleotide at the

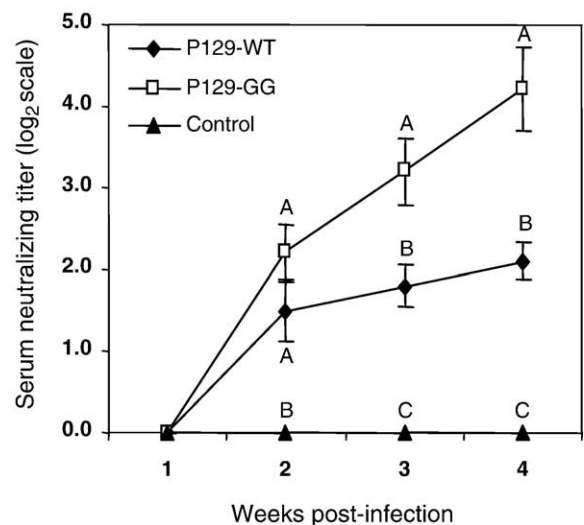


Fig. 5. Virus-neutralizing antibodies in the sera of pigs inoculated with wild-type virus or NLS-null mutant P129-GG virus. Neutralizing antibody titers are expressed on a log₂ scale. Error bars represent standard errors for each group of pigs at the indicated time. At each time point, means with different capital letters (A, B, or C) differ significantly ($P < 0.05$).

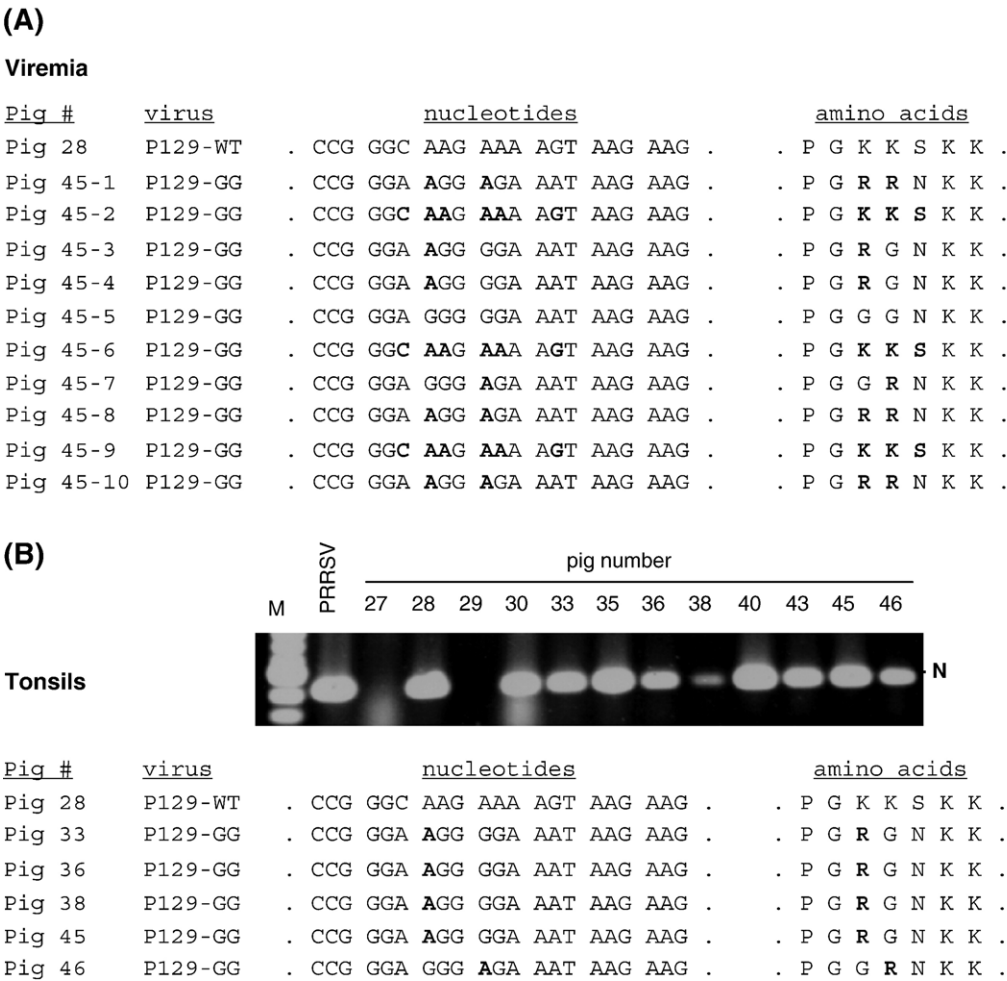


Fig. 6. The nucleotide (left) and deduced amino acid (right) sequences at the NLS motif from viral plaques obtained from pig45 infected with P129-GG virus (A). Plaque assays were carried out in Marc-145 cells using serum samples collected at 7 days post-infection from pig45. Ten plaques were randomly chosen, and total RNA was extracted from each plaque. The N gene was PCR-amplified and sequenced. (B) Persistence of PRRSV in the tonsils of infected pigs. The tonsils were taken from pigs at 28 days post-infection, and total RNA was extracted from the tonsil homogenates. The N gene was amplified by RT-PCR and visualized on a 1% agarose gel. 400-bp fragments are visible in the tonsils from infected pigs. Nucleotide and deduced amino acid sequences at the NLS locus of N gene products were determined and presented. Boldfaces indicate changed nucleotides and the corresponding amino acids. P129-WT denotes wild-type virus; P129-GG denotes the NLS-null mutant.

NLS locus, which led to an amino acid change from glycine to arginine at either position 43 or 44 (Fig. 6B). It is noteworthy that such a mutation to 43-PGRGNKK or 43-PGGRNKK represents a potentially functional ‘pat7’ NLS motif, implicating possible association of PRRSV persistence with the nuclear localization of N in the tonsils of these pigs.

N protein nuclear localization of revertants

Since mutations at the NLS locus were found from various plaques isolated in pig 45 (Fig. 6A) as well as from the tonsils of pigs infected with P129-GG (Fig. 6B), it was of particular interest to assess nuclear localization of reverted N proteins. A total of four different mutation patterns were identified during



Fig. 7. Nuclear localization of N proteins from the NLS reversions arising during viremia and persistence. The wild-type N gene in pCi-Neo, containing the wild-type NLS of 41-PGKKNKK, was mutated by site-directed mutagenesis to 41-PGGGNKK, 41-PGKKSCK, 41-PGRRNKK, 41-PGGRNKK, or 41-PGRRNKK. Each N gene construct was transfected to Marc-145 cells for 2 days, and the cells were immunostained using the N-specific MAb SDOW17 to determine the N protein nuclear localization. Nucleus (Nu) and nucleolus (No) are indicated by arrows.

viremia and persistence from NLS-null virus-infected pigs: 41-PGRGNKK, 41-PGGRNKK, 41-PGRRNKK, and 41-PGKSKK. These mutations were subsequently introduced to the wild-type N gene by site-directed mutagenesis. Four different reversion types of N were individually transfected into Marc-145 cells for 2 days, and the cells were stained with N-specific MAb SDOW17. All four types of reversions were able to translocate the N proteins to the nucleus and nucleolus (Fig. 7), demonstrating that all reversions regained a functional NLS. The function of 41-PGRGNKK was somewhat unexpected, as it does not conform to the previously defined “pat7” NLS motif (Rowland et al., 1999, 2003).

Discussion

The primary replication site for most RNA viruses is the cytoplasm of infected cells. During the past decade, however, some RNA virus proteins have been shown to localize to the nucleus and nucleolus (reviewed in Hiscox, 2003). It is postulated that these viruses have evolved to utilize nuclear functions of the cell to favor productive infections, to inhibit the synthesis of competing cellular macromolecules, or to modulate antigen presentation or innate immunity. A number of RNA virus proteins that are translocated to the nucleus and nucleolus have effects on the host cell nuclear functions through inhibition of host cell transcription, disruption of nucleocytoplasmic transport pathways, and induction of nuclear herniations in order to facilitate their replication and to inhibit host antiviral responses (Aminev et al., 2003a, 2003b, 2004; Chen et al., 2002; Hoyt et al., 2004; Petersen et al., 2000; Sharma et al., 2004). It has been difficult, however, to study such functions in vivo using replicating virus because of the need for reverse genetics systems to generate appropriate mutants. In one study using a mutant Semliki Forest virus (a member of the alphaviruses), in mice, a single amino acid change in the NLS locus of the nsP2 protein disrupted nsP2 nuclear transport and modified the neurovirulence of the virus (Fazakerley et al., 2002; Rikonen, 1996).

As with many other RNA viruses, PRRSV replicates in the cytoplasm, and the PRRSV proteins are largely distributed in the cytoplasm. The viral N protein, however, has been shown to localize both in the cytoplasmic and nucleolar compartments in porcine alveolar macrophages and Marc-145 cells (Rowland et al., 1999). The functional NLS has been identified in the N protein, and its nuclear interaction with fibrillarin has been characterized (Rowland et al., 2003; Yoo et al., 2003). In the present study, we investigated the biological importance of N protein nuclear localization during infection in cultured cells and in pigs. It was once speculated that the induction of CPE by PRRSV infection could be related to the nuclear localization of N protein (Rowland and Yoo, 2003). However, Marc-145 cells infected with the NLS-null mutant virus produced cytopathology indistinguishable from that of wild-type virus, but with lower viral titers and smaller size plaques. Our data demonstrate that nuclear localization of N is not related to PRRSV cytopathology and is not essential for production of infectious progeny.

Acute infection by PRRS virus causes viremia that is detectable commonly at 2 to 11 days post-infection, but up to 23 days in some pigs (Allende et al., 2000). In this experiment, the onset of viremia was detected as early as 4 days post-infection in infected pigs, but none of the pigs continued to harbor the virus in their sera by 14 days. The sensitivity of virus isolation from serum specimens may be limited to 2 weeks post-infection. Other methods of virus detection with greater sensitivity such as real-time RT-PCR may have been able to detect virus after 14 days but would not distinguish infectious virus from residual viral RNA. The shorter duration of viremia in P129-GG pigs probably reflects growth attenuation of the NLS-null virus.

PRRSV-infected pigs produce detectable IgG at 7 to 14 days post-infection with an increase to 4 weeks post-infection followed by decline and persistence for 1 year (Labarque et al., 2000; Rossow et al., 1994; Yoon et al., 1995). In our study, seroconversion was detectable by 10 days post-infection in all virus-infected pigs, but virus neutralizing antibody titers were significantly higher in P129-GG infected pigs by 3 weeks post-infection. A typical immune response to PRRSV in pigs is characterized by early production of non-neutralizing antibodies followed by the delayed appearance of neutralizing antibodies between 2 and 4 weeks post-infection, which then persist at low levels. Moreover, typical titers of serum neutralizing antibodies are between 2 and 12, titers which are considered unusually low in comparison with those induced by viruses of other families (Labarque et al., 2000; Loemba et al., 1996). The role of antibodies in protection is also uncertain since PRRSV can replicate and spread in pigs even in the presence of circulating antibodies (Loemba et al., 1996; Rossow et al., 1995). In contrast, the protective effects of passively transferred PRRSV-specific neutralizing antibodies have been demonstrated, suggesting that serum neutralizing antibodies may be the key component for protection from PRRSV infection (Lopez and Osorio, 2004; Osorio et al., 2002). PRRSV-specific antibody-dependent enhancement of infection has also been reported (Yoon et al., 1996). In the present study, pigs infected with P129-GG had detectable SN titers by 3 weeks post-infection; titers increased to 18 by 4 weeks. It is of interest to examine whether the increased SN titers in P129-GG infected pigs contribute to better protection against PRRSV infection. In the present study, the appearance of SN antibodies coincided with disappearance of viremia after 14 days of infection. Immune responses in the P129-GG infected pigs may have led to lower viral replication and faster clearance of virus from the serum.

Pig 45 exhibited responses distinct from those of other animals in the P129-GG group. Direct sequencing of viral plaques demonstrated that mutation of the P129-GG virus had indeed occurred in this pig. Mutant viruses contained at least one amino acid change within the NLS locus at positions 43 through 45 of N. Mutated N proteins were able to translocate to the nucleus and nucleolus, suggesting alterations of both genotype and phenotype of the null mutant during infection. Although these changes were extremely minor at the genomic sequence level (1 or 2 nucleotide changes out of the total

genome of 15,395 nucleotides), there were functional consequences during infection of the pig. Considering that the N gene is the most stable gene in PRRSV in pigs (Le Gall et al., 1998), the appearance of such reversions during the relatively short period of infection is surprising. The data suggest the presence of strong selection pressure at the NLS locus of N for functional NLS in the host.

PRRSV infection generally leads to virus persistence characterized by limited virus replication in the lymphoid tissues. Shedding of virus from persistently infected animals is a main factor which makes the control of PRRS difficult in the field (Allende et al., 2000; Wills et al., 1997). The mechanism of PRRS virus persistence is unknown. During persistence, structural genes are generally variable, with the exception of the N gene which is considered genetically stable (Allende et al., 2000). The genetic stability of the N gene has also been observed for EAV during persistence in stallions (Hedges et al., 1999). It is interesting to note that the EAV N protein also specifically localizes to the nucleus upon infection (Tijms et al., 2002). In the present study, both wild-type and P129-GG viruses persisted at least for 1 month in the tonsils of pigs. Surprisingly, all P129-GG viruses isolated from the tonsils of infected pigs harbored mutations at the NLS locus. In all cases, one or two nucleotide changes occurred, and these changes led to amino acid alterations from glycine to arginine at position 43 or 44 of N. The preferential mutation to arginine is explained by the fact that a single nucleotide change from 'G' to 'A' will code for arginine, whereas two nucleotide changes are required to code for lysine. No silent mutations were found in more than 18 plaques examined. The arginine mutation at position 43 or 44 reverted the NLS-null virus to a functional phenotype. Indeed, the N proteins containing an arginine mutation at 43 or 44 were all found to localize in the nucleus and nucleolus. The observation that 41-PGRGNKK was positive for NLS function was unexpected, since it deviates from the "pat7" motif definition of a proline followed within three residues by a segment containing three basic residues (K or R) out of four (Rowland et al., 1999, 2003). In order to accommodate all functional sequences, the NLS motif of North American genotype PRRSV N proteins is herein expanded and redefined as a "pat8" motif: a proline followed within three residues by a segment containing at least three basic residues (K or R) out of five. The corresponding sequences in isolates VR-2332 and P129 are 41-PGKKNKKK and 41-PGKKSKKK, respectively. The most common functional NLS revertant found in tonsils during the present study, 41-PGRGNKKK, conforms to the pat8 (but not the pat7) pattern. A review of sequences in Genbank reveals that nearly all North American PRRSV isolates conform to the pat7 motif, but there are three exceptions. Genbank accession numbers AF043951, AY209228, AY262352 describe field isolates with NLS sequences of 41-PGKNNKKK, 41-PGKKNMKR, and 41-PGKKNKKR, respectively. The pat8 motif encompasses these and all other known North American PRRSV N protein sequences (over 70 at the time of this analysis). Collectively, our data suggest a potential correlation of the N protein nuclear localization and viral persistence or virus fitness. N protein nuclear localization

may play an important role in evasion of host immune mechanisms by PRRSV.

Materials and methods

Cells, viruses, and antibodies

Marc-145 cells (a subclone of MA104 cells; Kim et al., 1993) and COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 8% fetal bovine serum (FBS; Gibco BRL), penicillin (100 U/ml), and streptomycin (50 µg/ml). Cells were maintained at 37 °C with 5% CO₂. Stocks of reconstituted viruses derived from infectious cDNA clones were prepared after three passages in Marc-145 cells. Titers of the viruses were determined by standard plaque assays. Plaque assays were carried out in duplicate in Marc-145 cells using 6-well plates of 35 mm in diameter per well. Virus-infected cell monolayers were overlaid with 0.8% agarose in DMEM and incubated at 37 °C. At 5 days post-infection, plaques were stained with 0.01% neutral red, and plaque numbers were determined. Monoclonal antibodies (MAbs) specific for N are described elsewhere (Nelson et al., 1993; Wootton et al., 1998). *E. coli* strains XL1-Blue (Stratagene) and DH5α were used as hosts for site-directed mutagenesis and general cloning, respectively.

Generation of NLS-deficient full-length PRRSV cDNA clones

To modify the NLS (41-PGKKSKK for P129 strain; 41-PGKKNNKK for PA8 strain) at positions 41 to 47 of the N protein, PCR-based site-directed mutagenesis was conducted to substitute codons for lysine residues at positions 43 and 44 (genomic nucleotide positions 14,999 to 15,004) to codons for glycine using pCi-Neo-N and the shuttle plasmid with the following primer pairs: for KK43/44GG mutation, KK43/44GG-Fwd (5'-GGCAAGGGACCGGGAGGGGGAAATAAGAAGAAA-AAC-3'; nucleotide positions 14,984 to 15,019) and KK43/44GG-Rev (5'-GTTTTTCTTCTTATTTCCCCCTCCCGGT-CCCTTGCC-3'; nucleotide positions 14,984 to 15,019), where underlines indicate codon changes for amino acid substitutions from 'KKS' to 'GGN'. PCR-based mutagenesis and screening of mutants were performed as described previously (Wootton et al., 2001). The cDNA cloning of the N gene from the PRRSV strain PA-8 into pCi-Neo (Promega) to produce pCi-Neo-N is described elsewhere (Wootton et al., 2002).

The shuttle plasmid pTB-shuttle-N-GG carrying the NLS mutation was digested with *Bsr*G I and *Spe* I, and a 2772-bp fragment was purified. The wild-type full-length genomic cDNA clone was digested with *Bsr*G I and *Spe* I, and the 2772-bp *Bsr*G I-*Spe* I fragment was replaced with the corresponding fragment obtained from the shuttle plasmid. The ligated full-length plasmid DNA was screened by *Xma* I digestion, and based on the *Xma* I digestion pattern, positive clones were selected. DNA manipulation and cloning were performed according to standard procedures (Sambrook and Russell, 2001). The selected clones were sequenced to confirm the presence of 'GGN' modification in the full-length genomic

cDNA clone. The resulting plasmid was designated pCMV-S-P129-GG.

Immunofluorescence staining

Marc-145 cells or COS-7 cells were seeded on microscope coverslips placed in 35-mm-diameter dishes and grown overnight to 70% confluency. The cells were transfected with 2 µg of plasmid DNA using Lipofectin according to the manufacturer's instruction (Invitrogen). At 48 h post-transfection, cell monolayers were washed twice in PBS and fixed immediately with cold methanol for 10 min at –20 °C. Cells were blocked using 1% bovine serum albumin in PBS for 30 min at room temperature and then incubated with N-specific MAb SDOW17 for 2 h. After washing five times in PBS, the cells were incubated for 1 h at room temperature with goat anti-mouse secondary antibody conjugated with Alexa green dye (Molecular Probes). The coverslips were washed five times in PBS and mounted on microscope glass slides in mounting buffer (60% glycerol and 0.1% sodium azide in PBS). Cell staining was visualized using a fluorescent microscope (model AX70, Olympus).

Production of virus from full-length cDNA clones

Marc-145 cells were seeded in 35-mm-diameter dishes and grown to 70% confluency. Cells were transfected for 24 h with 2 µg of the full-length cDNA plasmid using Lipofectin. Transfected cells were incubated for 5 days at 37 °C in DMEM supplemented with 8% FBS. The culture supernatants were harvested at 5 days post-transfection and designated 'passage-1'. The passage-1 virus was used to inoculate fresh Marc-145 cells, and the 5-day harvest was designated 'passage-2'. The 'passage-3' virus was prepared in the same way as for passage-2. Each passage virus was aliquoted and stored at –80 °C until use.

RT-PCR and sequencing of N gene

Viral RNA was extracted from either supernatants or lysates of infected cells using the QiaAmp viral RNA mini-kit (Qiagen). To remove any possible contamination of DNA in the RNA preparations, samples were treated with 1 unit of RQ DNase I (Promega) per microgram at 37 °C for 30 min in 50 mM Tris–HCl [pH 7.5] and 1 mM MgCl₂. First-strand cDNA synthesis was performed by Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) using the reverse primer ORF7-Rev (5'-AGAATGCCAGCCCATCA-3'). The N gene was amplified by *Taq* DNA polymerase (Invitrogen) using ORF7-Fwd (5'-CCTTGTCAAATATGCCAA-3') and ORF7-Rev under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of: denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were analyzed by 1% agarose gel electrophoresis. Amplified products were purified using the PCR purification kit (Qiagen) and sequenced.

Preparation of virus inocula

Due to the low titer of P129-GG virus, the 'passage-3' virus was concentrated for pig experiments. Cells were plated in 50 dishes of 100-mm-diameter and grown overnight to 70% confluency. The cells were infected with the 'passage-2' P129-GG virus at a multiplicity of infection (MOI) of 0.1 for 1 h at 37 °C. Following infection, 5 ml of fresh medium containing 8% FBS was added, and incubation continued for 4 days. When extensive CPE had developed, culture supernatants were harvested and pooled, and cell debris was removed by a low-speed centrifugation for 10 min at 1350 × *g*. The supernatant was then pelleted by a high-speed centrifugation for 90 min in a JA-14 rotor (Beckman) at 30,000 × *g* and 4 °C. The pellet was resuspended overnight in one tenth of the original volume at 4 °C. Recovery of infectivity of the concentrated virus was determined by plaque assay on Marc-145 cells, and the virus stock was stored at –80 °C until use.

Experimental infection of pigs

Twenty-one York–Landrace piglets were obtained from a PRRSV-free swine herd at 5 weeks of age. Piglets were pre-screened serologically for evidence of exposure to PRRSV and *Mycoplasma hyopneumoniae* and were acclimatized for 7 days in an isolation facility prior to infection. Animals were randomly allotted to three groups, seven piglets per group, and animals within the same group were housed in the same room. Throughout the study, pigs were fed an age-appropriate non-medicated diet and provided with water ad libitum. On the day of challenge, the PRRSV stocks were diluted to make 2.5×10^4 PFU/ml, and each animal was infected with a total of 5×10^4 PFU by instillation of 1 ml in each nostril. After infection, an aliquot of each inoculum was back-titrated by plaque assay to confirm the dose of infection. Clinical signs for general condition, depression, loss of appetite, coughing, sneezing, and respiratory distress were monitored and rectal temperatures were measured daily in all pigs for the first week. All pigs were weighed upon arrival at the site and at necropsy. Blood samples were taken on days 0, 4, 7, 10, 14, 21, and 28 post-infection for virus isolation and serology. Serum samples were aliquoted and stored immediately at –80 °C until use. Two pigs from each treatment group were euthanized at 14 days post-infection to assess lung lesions, and the remaining pigs were euthanized at 28 days post-infection. At necropsy, the percent consolidation of each lung lobe was estimated to calculate total lung lesion severity, and a lung sample from a representative lesion was taken and immersed in formalin for histopathological examination. At Day 28 post-infection necropsy, tonsil samples were collected from remaining pigs for evaluation of viral RNA via RT-PCR. The infection study and necropsies were performed in the animal isolation facilities of the Ontario Ministry of Agriculture and Food, located at the Ontario Veterinary College, University of Guelph (Guelph, Ontario, Canada). The study protocol was approved by the University of Guelph Animal Care Committee.

Viremia, ELISA, and serum neutralization assays

Viremia was measured by plaque assay in Marc-145 cells in duplicate. Cells were infected with 0.1 ml of 10-fold serial dilutions of sera collected at indicated days. The number of plaques was counted for each dilution, and virus titers were expressed as PFU per milliliter. Antibody titers were determined at the Animal Health Laboratory of the Laboratory Services Division, University of Guelph, using the commercially available PRRSV antibody detection kit (HerdCheck PRRS; IDEXX, Westbrook, Maine), accordingly to the manufacturer's instruction. ELISA results were expressed as a ratio of the optical density for the sample compared to the optical density for the positive control (S/P ratio). According to the assay, an S/P ratio greater than 0.4 is considered positive for the presence of PRRSV antibodies.

Serum neutralization tests were performed in Marc-145 cells in a 96-well microtiter plate format using the wild-type PRRSV P129-WT virus. The 'passage-3' P129-WT virus stock was diluted in DMEM to make 200 PFU in a 50 µl volume. The diluted virus was mixed with 50 µl of 2-fold dilutions of individual sera obtained from 0, 1, 2, 3, and 4 weeks post-infection. The mixtures were then incubated for 1 h at 37 °C, and approximately 2×10^5 Marc-145 cells in 100 µl were added to each well. After 5 days of incubation, individual wells were scored by CPE. Serum neutralization tests were conducted four times, each time in duplicate.

Isolation of revertants

To identify mutant virus arising during infection, plaque assays were performed using serum samples taken at 7 days post-infection. When plaques developed, individual plaques were picked using pipettes, and the agarose plugs were resuspended in 1 ml of DMEM. Total RNA was extracted from the plaque suspension using TRIzol reagent (Invitrogen). The plaque suspensions were also used to infect Marc-145 cells for virus isolation. To identify mutants during persistence, tonsils taken at necropsy were homogenized in PBS, and total RNA was extracted using TRIzol. RT-PCR was then conducted to amplify the N gene followed by nucleotide sequencing.

Testing NLS function of revertants

To examine whether N proteins of revertants were capable of nuclear localization, mutations identical to the reverted sequences were introduced into plasmid pCi-Neo-N-KK/43.44/GG using the following primer pairs: for 41-PGRGNKK, G43R-Fwd (5'-GGGACCGGGAAGGGGAAATAAG-3': nucleotide positions 14,989 to 15,010) and G43R-Rev (5'-CTTATTTCCCTTCCCGGTCCC-3': nucleotide positions 14,989 to 15,010); for 41-PGGRNKK, G44R-Fwd (5'-GGA-CCGGGAGGGAGAAATAAGAAG-3': nucleotide positions 14,990 to 15,013) and G44R-Rev (5'-CTTCTTATTTCTCCTCCCGGTCC-3': nucleotide positions 14,990 to 15,013); and for 41-PGRRNKK, G43/44R-Fwd (5'-GGCAAGG-

GACCGGGAAGGAGAAATAAGAAGAAAAAC-3': nucleotide positions 14,989 to 15,019) and G43/44R-Rev (5'-GTTTTCTTCTTATTTCTCCTTCCCGGTCCCTTGCC-3': nucleotide positions 14,989 to 15,019), where underlines indicate codon changes for amino acid substitutions from GG to RG, GR, or RR.

Statistical analysis

Statistical calculations were carried out using SAS version 9.1 (SAS Institute Inc., Cary, North Carolina). Comparisons of virus titers and virus neutralization antibody titers were carried out using logarithmically transformed data by analysis of variance (ANOVA) with Duncan's multiple comparison test used when the ANOVA test indicated significant differences. Comparisons of S/P ratios generated by the IDEXX PRRSV ELISA were made using the Kruskal–Wallis non-parametric test.

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